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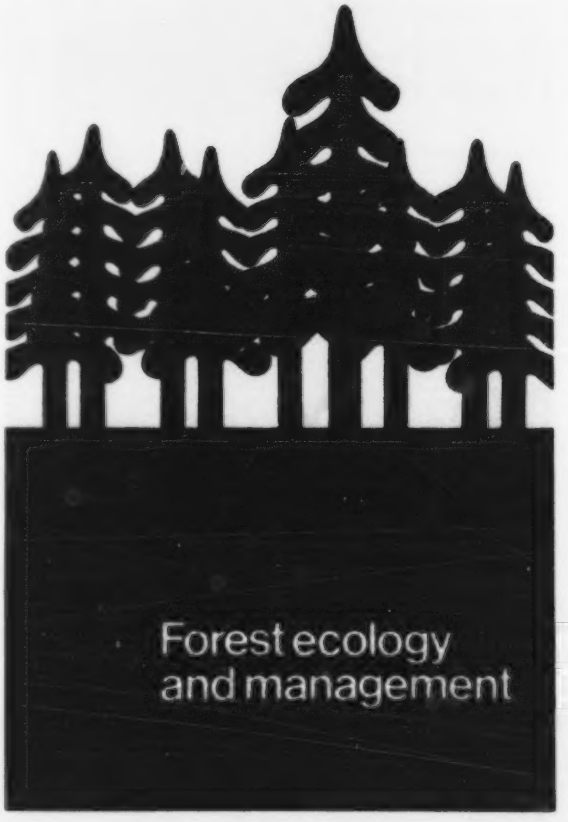

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HAPLOID PLANT PRODUCTION THROUGH ANTHER CULTURE IN POPLARS

R.H. HO and Y. RAJ

Ontario Tree Improvement and Forest Biomass Institute, Ontario Ministry of Natural Resources, Maple, Ont. LOJ 1EO (Canada)

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ABSTRACT

Ho, R.H. and Raj, Y., 1985. Haploid plant production through anther culture in poplars. *For. Ecol. Manage.*, 13: 133—142.

Haploids plantlets were produced from pollen through anther culture in four out of seven poplar (*Populus*) species and hybrids. Anthers with pollen at the mononucleate stage were incubated on Murashige and Skoog's medium supplemented with kinetin and 2,4-dichlorophenoxyacetic acid. After a 50-day incubation in darkness, calli evolving from the anther cavities were transferred on to the medium supplemented with benzylaminopurine and naphthaleneacetic acid. Organogenesis occurred in 3 months after exposure to light for 16 h per day. The adventitious shoots were transferred to a medium containing naphthaleneacetic acid where they rooted in 2 weeks. One artificial hybrid, *P. maximowiczii* × *deltoides*, produced some haploid plants with parental leaf morphology and others with intermediate forms. Most calli produced plantlets with the haploid chromosome number of 19 and some regenerated plantlets with chromosome numbers ranging from 11 to 38.

INTRODUCTION

Poplar (*Populus*) species are dioecious; staminate and pistillate flowers are borne on different plants. Since they are dioecious, homozygous lines can only be obtained through sib-mating. However, the long juvenile phase and many generations of inbreeding via sib-mating makes the production of homozygous lines impractical. The problem is also compounded by incompatibility and inbreeding depression.

Since the discovery of haploid plantlet induction through anther culture (Guha and Maheshwari, 1964), isogenic pure lines have become attainable by diploidization of the haploid. The pure line differs from the homozygous line in that the latter still contains some degree of heterozygosity.

Successful haploid plantlet induction through anther or megagametophyte culture in tree species was first reported by Wang et al. (1975) and Bonga (1977). To date, such induction has been successful in 24 species and hybrids

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of the genera *Aesculus* (Radojevic, 1978), *Betula* (Huhtinen, 1978), *Hevea* (Chen et al., 1979, 1982), *Populus* (Wang et al., 1975; Heilungkiang Inst. For., 1975; N.E. For. Acad., 1977; Zhu et al., 1980; Ho et al., 1983), and *Picea* (Bonga, 1977; Huhtinen et al., 1981; Simola and Honkanen, 1983). The present paper describes the culturing of poplar anthers and the production of haploid plantlets.

MATERIAL AND METHODS

Seven poplar species and hybrids were selected for the study (Table 1). The trees, ranging from 17 to 25 years of age, were growing in the arboretum at the Ontario Tree Improvement and Forest Biomass Institute, Maple, Ont., Canada.

Branches bearing staminate catkin buds were collected before natural anthesis and were placed in water in a laboratory at room temperature to force them to flower. A few anthers were squashed daily with acetocarmine to determine the stages of microsporogenesis and pollen formation.

When the pollen mother cells were at the tetrad stage, or pollen grains were at the mononucleate or dinucleate stage, discs (fan shape, about 4 mm in length and 2 mm in height) bearing stamens were removed from the

TABLE 1

Callus formation, plantlet production, and chromosome number of the plantlets in *Populus* species and hybrids

Species	Clone number	Somatic callus	Callus from anther cavity	Plantlet	Chromosome number
P. angulata Ait. × *simonii* Carr.	ANS-1	+	+	-	-
P. balsamifera L. × *deltoides* Marsh. (*P. jackii* Sarg.)	jackii-1	+	+	+	19—26
P. deltoides Marsh.	D-8, D-9	+	+	+	19—38
P. × *euramericana* cv. *eugenei* Schelle	Carolina-1	+	+	-	-
P. maximowiczii Henry × *deltoides* Marsh.	MD-1	+	+	+	11—38
P. nigra L.	N-166	+	+	+	19—38
(*P. trichocarpa* Hook. × *angulata* Ait.) × (*P. balsamifera* L. × *deltoides* Marsh.)	IE-33	+	+	-	-

catkins and surface sterilized with 10% 'Javex' bleach solution (0.6% sodium hypochlorite) for 10 min. After rinsing 3 times with sterile distilled water, the anthers were placed on a culture medium. About 100 anthers were incubated on each petri dish (60 × 20 mm) and 10 dishes were used for each incubation per species or hybrid.

Three basic media were used based on Murashige and Skoog's (MS) basal medium (macronutrients, micronutrients, and vitamins) (1962).

(1) A callus-inducing medium (CIM) consisting of two forms; one of which (CIMa) was the MS medium supplemented with various combinations of different concentrations (0—10 mg/l) of the auxins 2,4-dichlorophenoxyacetic acid (2.4-D), and naphthaleneacetic acid (NAA) and the cytokinins kinetin and benzylaminopurine (BAP); the other (CIMb) was similar to the CIMa except that the FeEDTA was reduced to one fifth.

(2) An organ-inducing medium (OIM), including two forms (OIMa, OIMb), which was the same as the CIM except that different combinations of cytokinins and auxins were used.

(3) A rooting medium was composed of the MS medium in which all the macronutrients were reduced to one-half strength and various combinations of different concentrations of NAA and indolebutyric acid (IBA) were added.

Three percent sucrose was used for all the media except the rooting medium, which contained 2% sucrose. All the media had 0.6% agar and the pH was adjusted to 5.8 before autoclaving at 1 kg cm^{-2} for 15 min.

Anthers were incubated on the CIM and calli on the OIM in petri dishes (60 × 20 mm). The calli with or without adventitious shoots were transferred monthly on to a fresh medium in jars (70 × 85 mm).

Adventitious shoots of about 3 cm in height were rooted in the rooting medium in test tubes (25 × 150 mm). The cultures were maintained at 25°C during the day and 15°C at night. The anther cultures were kept in the dark for 2 months and the callus culture and rooting shoots in a 16-h photoperiod. During the 2 months of incubation, the cultures were taken out of the incubator briefly once a week for examination of callus development under a dissecting microscope. The photosynthetic photon flux density in the incubators averaged 30 μEs^{-1} m^{-2}.

Plantlets were cleaned by removing the agar medium and were then potted in a soil mixture of equal amounts of perlite and peat. They were kept in a mist house with a relative humidity of over 90% for at least 2 weeks before transferring to a greenhouse. The potted plants were established in the nursery in the spring.

Ploidy level of the calli and plantlets was determined as follows. Leaves from young adventitious shoots and root tips were treated with 0.1% colchicine for 3 h and then hydrolyzed in 1 *N* HCl at 60°C for 10 min, then they were squashed and stained in the aceto-carmine solution.

RESULTS

Callus induction

Poplar anthers (Table 1) produced calli in 2 weeks. The callus induction frequency was as high as 100% in some dishes after about 50 days of culture (Fig. 1). All the calli were amorphous and most were regenerated from either the anther cell wall, connective tissue or filament. Some protruded from the anther cavity through a split in the absicission layer of the anther cell wall after a month of incubation. The latter could have originated from either pollen, the anther inner cell wall, or tapetal cells. Normally, one callus was observed in each cavity but in some cases two calli were found, each at opposite ends of the cavity (Fig. 2).

Fig. 1. Amorphous calli from the somatic tissues in *P. maximowiczii* × *deltoides* (× 1.2).
Fig. 2. Two calli in an anther cavity in *P.* × *euramericana* cv. *eugenei* (× 3).

Upon exposing the culture to light, almost all calli evolving from the anther cavities turned green. In contrast, only a few regenerated elsewhere turned green. All the green calli evolving from the anther cavities were regenerated from anthers with pollen at the mononucleate stage. No green callus was produced from anthers with pollen mother cells at the tetrad stage or with pollen at the binucleate stage. Based on the number of anthers incubated, the induction frequency for green calli was less than 2% for all the species except the artificial hybrid of *P. maximowiczii* × *deltoides* which had a frequency of 4.8%.

For most species and hybrids, the CIMa supplemented with 2 mg/l of 2,4-D and 1 mg/l of kinetin was found best for callus induction from both somatic tissue and pollen grains, while the hybrid *P. maximowiczii* × *deltoides* responded best in the CIMb with the same supplement.

Organogenesis in calli

No organogenesis occurred in either non-green or green calli regenerated from the somatic tissues after 2 years culture on either the CIM or the

OIM. However, most calli evolving from the anther cavities produced either adventitious shoots, roots or both in 3 months after the calli had reached 5 mm in diameter and were transferred onto the OIM (Figs. 3 and 4). In some cases, some sections of the roots thickened to produce calli which later regenerated some adventitious shoots (Fig. 5).

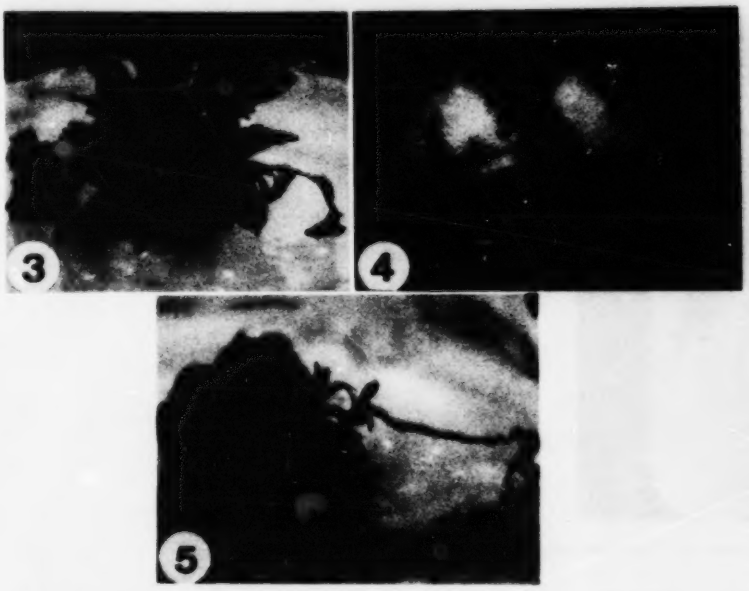

Fig. 3. Callus with adventitious shoots in *P. jackii* (× 1.2).

Fig. 4. Callus with adventitious roots in *P. deltoides* (× 3).

Fig. 5. Adventitious root with a callus (arrow) in *P. nigra* (× 1.2).

In all the media used to induce organogenesis, the OIMa supplemented with 1 mg/l of BAP and 0.2 mg/l of NAA was found best for all the poplars, except for *P. maximowiczii* × *deltoides* which performed best on the OIMb with the same supplement. Regeneration of the adventitious shoots was most frequent in calli maintained on media containing only one-tenth of the BAP concentration. Of the seven poplars producing adventitious shoots, *P. deltoides*, *P. jackii*, *P. maximowiczii* × *deltoides* and *P. nigra* had shoots that could be excised for rooting. The rest of the poplars had either pale green or albino shoots which generally died before they could be removed for rooting.

Rooting adventitious shoots

The adventitious shoots rooted after 2 weeks in the rooting medium (Fig. 6). The best rooting percentage (90%) occurred in the medium con-

taining 0.02 mg/l of NAA, the next best (70%) in the medium containing 0.02 mg/l of IBA. The rooted shoots (plantlets) grew to about 5 cm in 2 weeks. The plantlets of *P. deltoides*, *P. jackii*, *P. maximowiczii* × *deltoides*, and *P. nigra* were transferred to pots with soil and about 90% of the plantlets survived the transfer.

Fig. 6. A plantlet in *P. maximowiczii* × *deltoides* (× 1).

Morphological variation

Little morphological variation was observed in *P. deltoides*, *P. jackii* and *P. nigra* plantlets established in soil. However, some variations were observed in plantlets regenerated from *P. maximowiczii* × *deltoides*. Differences in leaf morphology were observed in plants regenerated from different calli but few morphological differences were found in plants arising from the same callus. Most calli produced plants with the leaf form of either the pistillate parent (ovate) or the staminate parent (deltoid) (Figs. 7 and 8). Some had either a lanceolate or a spatulate leaf form (Figs. 9 and 10).

Karyotype analysis

Chromosome counts showed that the green calli evolving from the anther cavities had been induced from pollen. The adventitious shoots or roots obtained from these calli generally contained the haploid chromosome number of 19 (Fig. 11). Some of the calli produced plantlets with a chromosome number ranging from 19 to 38 (Table 1). One callus from *P. maxi-*

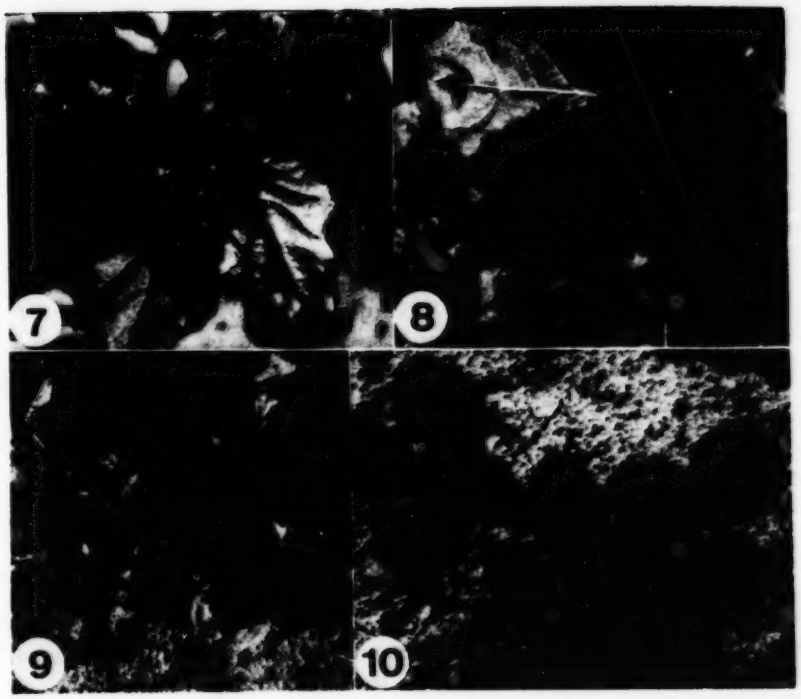

Fig. 7. Ovate leaves in *P. maximowiczii* x *deltoides* (x 0.3).

Fig. 8. Deltoid leaves in *P. maximowiczii* x *deltoides* (x 0.3).

Fig. 9. Lanceolate leaves in *P. maximowiczii* x *deltoides* (x 0.3).

Fig. 10. Spatulate leaves in *P. maximowiczii* x *deltoides* (x 0.3).

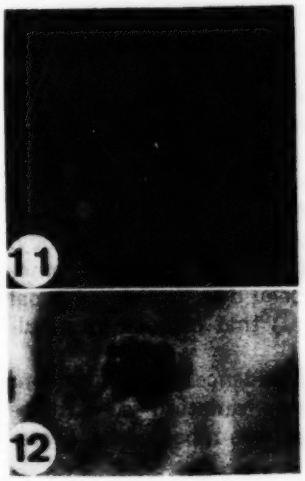

Fig. 11. Nineteen (haploid) chromosomes in *P. maximowiczii* x *deltoides* (x 800).

Fig. 12. Eleven chromosomes in *P. maximowiczii* x *deltoides* (x 800).

mowiczii × *deltoides* was found producing plantlets with chromosome numbers ranging from 11 to 22 (Fig. 12).

DISCUSSION

The best stage in microsporogenesis and pollen formation in angiosperms has been well defined for haploid callus induction (Maheshwari et al., 1980). The maximum response is usually obtained when pollen in anthers is at the mononucleate stage prior to mitosis. The same stage was found best for the poplar species in the present study. A similar conclusion was reported for horse chestnut (*A. hippocastanum*) (Radojevic, 1978), rubber plant (*H. brasiliensis*) (Chen et al., 1979), poplar species (Zhu et al., 1980), and American elm (*Ulmus americana*) (Redenbaugh et al., 1981).

Organogenesis in calli regenerated from the pollen of tree species has two basic forms: (1) production of embryoids, and (2) regeneration of either adventitious shoots or roots. The former has been reported in horse chestnut (Radojevic, 1978) and rubber plant (Chen et al., 1979), and the latter in European birch (*B. pendula*) (Huhtinen, 1978), and poplar species (Zhu et al., 1980).

Embryoids were formed in horse chestnut and rubber plant when calli were cultured on the MS medium containing kinetin and 2,4-D. In contrast, a proliferation of calli without organogenesis occurred when poplar calli were incubated on the above medium, but adventitious shoots were formed after the calli had been transferred onto the medium containing BAP and NAA (Zhu et al., 1980).

Seven poplar species and hybrids studied produced adventitious shoots or roots, but only four of these regenerated shoots that could be rooted and established in soil. Many shoots or plantlets of the seven species and hybrids regenerated from calli did not survive in either rooting medium or soil. This may have been attributed to (1) the genetic load of deleterious genes on the haploid or homozygous diploid genomes, and (2) the incidence of aneuploidy, since aneuploidy is usually fatal (Yeoman and Forche, 1980).

Variation in leaf morphology of the regenerated plants was slight in the pure species, *P. deltoides* and *P. nigra*, and a natural hybrid, *P. jackii*. The lack of variation in *P. jackii* could have been due to (1) the low number of plants regenerated, and (2) introgression that could have occurred in this hybrid. On the contrary, plants regenerated from the artificial hybrid, *P. maximowiczii* × *deltoides*, showed some variation in leaf morphology. This may have been the result of an independent assortment of chromosomes in meiosis. The variation could also have been due to chromosomal aberrations (cryptic or gross chromosome changes) that arose during culture.

Cultured plant cells generally exhibit a high incidence of spontaneous and heritable variation (Meins, 1983). Within-clone variation in chromosome numbers in in vitro regenerated poplar plants was reported by Lester

and Berbee (1977). In the present study, a similar variation was found in plantlets regenerated from one of the calli. These plantlets contained chromosome numbers ranging from 11 to the diploid number of 38. This dipioid chromosome number could have resulted from spontaneous diploidization in culture. Whether there is a correlation between the chromosome number and the variation in leaf morphology requires verification through karyotype analysis.

Haploid induction through anther culture has made the production of isogenic pure lines possible. In vitro culture also induces destabilization in cell genetics. This could have some important practical consequences since the culture would provide novel forms of variation.

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